Master's Thesis

Diversity of GABAergic Cell Types in the Developing Mouse Interpeduncular Nucleus

By

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Tiedekunta Bio- ja ympäristötieteellinen tiedekunta		Koulutusohjelma Neurotieteen maisteriohjelma
Tekijä Anniina Tervi		
Työn nimi GABAergisten hermosolujen monimuotoisuus hiiren interpedunkulaarisen tumakkeen kehityksessä		
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Tiivistelmä

Erilaisten hermosolujen kirjo luo perustan aivojen toiminnalle. Hermosolujen erilaisten osapopulaatioiden kehitys sekä hermosolujen erityisominaisuudet keskushermostossa ovat vielä osittain epäselviä. Näiden kehitysbiologisten asioiden ymmärtäminen voisi edistää aivorungon tumakkeiden solutyyppien rakenteen ja toiminnan kartoitusta. Aiemmat työt ovat osoittaneet, että pieni alue aivojen takaosassa (rV2) tuottaa sekä eksitatorisia (glutamatergisiä) että inhibitorisia (GABAergisiä) hermosoluja, jotka liittyvät aivorungon monoaminergisiin tumakkeisiin (Lahti *et al*., 2016).

Tässä maisterintutkielman tutkimusprojektissa tutkittiin Gsc2-transkriptiofaktoria ilmentävää hermosolujen osapopulaatiota aivorungon interpedunkulaarisessa tumakkessa. Tutkimusprojekti pohjautuu aikaisempiin tuloksiin yksittäisten solujen mRNA-sekvensoinnissa E13.5 ikäisillä hiirillä. Aiemman tutkimuksen sekvensointitulosten perusteella Gsc2:a ilmentävät hermosolut ovat GABAergisiä interneuroneita ja ovat lähtöisin rV2-osasta rombomeeri 1 alueelta taka-aivoista. Tässä projektissa tutkittiin myös Sall3-transkriptiofaktorin ja Gsc2-transkriptiofaktorin yhtenäistä alueellista ilmentymistä hiirisikiöiden aivorungon kehityksen aikana. Ennalta mainittujen lisäksi tutkimuksessa tutkittiin myös Notch-signaloinnin roolia solun välittäjäaineidentiteetin valitsemisessa GABAergisen ja glutamatergisen solutyypin välillä rV2-alueen hermosolujen kantasoluissa. Aiemman tutkimuksen sekvensointitulosten validointi suoritettiin tutkimuksessa käyttäen immunohistokemiallisia ja in situ hybridisaatio -menetelmiä E12.5 ja E15.5 ikäisillä hiiren sikiöillä.

Tutkimusprojektin tulokset tukevat aiemman tutkimuksen tuloksia Gsc2:a ilmentävien solujen alkuperästä rombomeeri 1 alueelta ja lisäksi osoittivat näiden kyseisten hermosolujen olevan tyypiltään GABAergisiä soluja. Sall3 ja Gsc2 -transkriptiofaktoreiden yhteistä ilmentymistä rV2-alueella tai interpedunkulaarisessa tumakkeessa ei voida tässä tutkimuksessa saatujen tulosten perusteella todentaa. Lisäksi tutkimuksessa saatujen tulosten mukaan Notch-signaalin estäminen rV2-alueella vähensi GABAergisten hermosolujen erilaistumista samalla alueella. Tutkimuksen tuloksiin viitaten voidaan todeta, että Notch-proteiini vaikuttaa GABA-välittäjäaineen valitsemiseen hiirten aivorungon hermosolujen kehityksen aikana.

Tutkimuksessa saadut tulokset osoittavat myös, että *Gsc2* voisi toimia rombomeeri 1 alueelta syntyvien GABAergisten interneuronien sekä interpedunkulaarisen tumakkeen takaosan hermosolujen merkkigeeninä. Lisäksi Notch-signaloinnista saadut tulokset voivat auttaa erilaisten mekanismien löytämisessä hermosolujen välittäjäaineidentiteettiin liittyen. Jatkotutkimusta ajatellen aikapisteitä ja GABA- sekä glutamatergisiä merkkigeenejä tulisi lisätä tutkimuksessa saatujen tulosten tukemiseksi.

Avainsanat
Goosecoid homeobox 2, Notch, interpedunkulaarinen tumake, GABAerginen solu, kehitysbiologia

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Abstract

The diversity of different neuronal types lays the foundation for different functions in the brain. The development of different subpopulations and special features of neurons in the central nervous system are still partly unknown. Finding answers to these developmental issues could help in the process of characterisation of cell types and mapping of neuronal networks between the brainstem nuclei in the brain. Previous studies have shown that a ventrolateral neuroepithelial domain in the anterior hindbrain, rV2, produces excitatory (glutamatergic) and inhibitory (GABAergic) neurons, which are related to monoaminergic nuclei in the brainstem (Lahti *et al*., 2016).

In this master's thesis project, the development of a subpopulation of neurons expressing Gsc2 transcription factor in the interpeduncular nucleus was studied. This project was based on single-cell RNA sequencing results conducted in E13.5 mice. Predicted by single-cell RNA sequencing results, *Gsc2* expressing cells are GABAergic interneurons and originate from the rV2 domain of the rhombomere 1 region in the hindbrain. Co-expression pattern with another transcription factor Sall3 with Gsc2 during development was also addressed in the study. Furthermore, the role of Notch signalling in the binary cell fate decision between GABAergic and the glutamatergic fate of rV2 neurons was investigated. Validation of single-cell RNA sequencing results was performed using in situ hybridisation and immunohistochemistry methods with mice embryos at the age of E12.5 and E15.5.

This study verified previously shown origin of *Gsc2* expressing cells to the rhombomere 1 region and in addition, showed that *Gsc2* expressing cells are GABAergic. Co-expression pattern of *Gsc2* with *Sall3* neither in the rV2 domain nor in the interpeduncular nucleus was seen in our results. In the rV2 domain, the depletion of Notch signalling decreased the expression of differentiating GABAergic neurons. This indicates that Notch has a role in GABAergic neurotransmitter identity during the development of brainstem neurons in mice.

Based on our results, *Gsc2* could be used as a lineage marker for GABAergic interneurons originating from the rhombomere 1 region and as a marker for a subpopulation of the interpeduncular nucleus. Furthermore, results from the role of Notch signalling could help in discovering the mechanisms related to the determination of neurotransmitter identity in rV2 neurons. Further investigations, in different developmental time points and with additional markers, are needed to verify these results.

Keywords
Goosecoid homeobox 2, Notch, Interpeduncular nucleus, developmental biology, GABAergic neuron

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Additional information
This project was conducted in the Laboratory of Interneuron Development, led by Kaia Achim:
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Abbreviations

5-HT – 5-hydroxytryptamine

CNS – Central nervous system

DAPI – 4',6-diamidino-2-phenylindole

Dgcr14 – DiGeorge syndrome critical region 14

E – Embryonic day

EEG – Electroencephalography

FR – Fasciculus retroflexus

GABA – γ-aminobutyric acid

Gad – Glutamic acid decarboxylase

Gata2 – GATA-binding factor 2

Gata3 – GATA-binding factor 3

GFP – Green fluorescent protein

Gsc2 – Gooseceoid homeobox 2

Hes – Hairy and enhancer of split

IHC – Immunohistochemistry

IPN – Interpeduncular nucleus

IPc – Caudal Interpeduncular nucleus

IPl – Lateral Interpeduncular nucleus

ISH – In situ hybridisation

Mash1 – Mammalian achaete-scute homolog-1

MHb – Medial habenula

mRNA – Messenger ribonucleic acid

nAChRα5 – Nicotinic acetylcholine α5-receptor

NICD – Notch intracellular domain

NTE – NaCl-Tris-HCl-EDTA

Otp – Orthopedia homeobox

Otx2 – Orthodenticle homeobox 2

PAX7 – Paired box 7

PBS – Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PFA – Paraformaldehyde

POD – Peroxidase

Psen1 – Presenilin 1

R1 – Rhombomere 1

REM – Rapid eye movement

RFP – Red fluorescent protein

RNA – Ribonucleic acid

rV2 – rhombencephalic ventral 2

Sall3 – Spalt like transcription factor 3

scRNAseq – single-cell RNA sequencing

SDS – Sodium dodecyl sulphate

SNpr – Substantia nigra pars reticulata

SSC – Saline sodium citrate

Tal1 – T-Cell acute lymphocytic leukaemia protein 1

TEA – Triethanolamine

V2aIN – Ventral 2 excitatory interneuron

V2bIN – Ventral 2 inhibitory interneuron

Vglut2 – Vesicular glutamate transporter 2

Vsx2 – Visual system homeobox 2

Wt – Wild type

Introduction

Interpeduncular Nucleus: Location and Development

Interpeduncular nucleus (IPN) is a nucleus located in the mammalian brainstem, more specifically in the posterior part of the interpeduncular fossa spanning across the median floor plate of the midbrain to the most rostral part of the hindbrain (Lorente-Cánovas *et al.*, 2012). It is a conserved structure among vertebrates from birds to mice and humans (Lorente-Cánovas *et al*., 2012). The composition of the IPN is well studied, and the main cell groups and projections of the nucleus are, in most parts, revealed (structure and projections studied, for example, by Shibata & Suzuki, 1984; Groenewegen *et al*., 1986; Barr *et al.*, 1987; Lorente-Cánovas *et al*., 2012; Quina *et al*., 2017). IPN is known to be connected to multiple different brain regions. A significant part of inputs to the IPN are derived from the medial habenula (MHb) reaching the IPN through the fasciculus retroflexus (FR) (Herkenham & Nauta, 1979). Neuronal projections from the IPN, on the other hand, reach areas such as the raphe nuclei, dorsal tegmental area, hippocampus, hypothalamus and multiple other regions in the brainstem (Groenewegen *et al*., 1986). IPN is composed of different compartments and different neuronal subtypes. Cells in the IPN are diverse with different identities such as γ-aminobutyric acidergic alias GABAergic cells, glutamatergic cells and serotonergic cells (Hamill *et al*., 1984; Hsu *et al*., 2013; Quina *et al*., 2017). Nevertheless, the nucleus seems still to have some unique and unrevealed neuron types (Funato *et al*., 2010).

During development, the different locations of the subnuclei of the IPN are formed between E11 and E15 (embryonic day) in rats (Lenn & Bayer, 1986). In rodents, the architecture of the different subnuclei of the IPN is distinguished by various cytological features, projections and neurochemicals (Groenewegen *et al*., 1986). Through the course of development, neuronal precursor cells of the IPN tend to migrate – following the rostral-ventral migration pattern – from the hindbrain neuroepithelium along both sides of the midline ventrally towards the midbrain and turn laterally in their rightful position to form the IPN (Lenn & Bayer, 1986). By using neuron birth dating analysis already in the 1980s, it was shown that the rostral parts of the IPN develop later compared to other subnuclei in that region (Lenn & Bayer, 1986). The development of different subpopulations of cells in the IPN has been studied, for example, in chicken and mice (Lorente-Cánovas *et al*., 2012; Moreno-Bravo *et al*. 2014). Molecular marker expression, such as the expression of *Nkx6.1*, *Otp*,

Otx2 and PAX7, determines the different domain borders of the avian IPN (Lorente-Cánovas *et al*., 2012). Furthermore, the cells to the IPN originate from different domains of rhombomere 1 (R1) and the isthmus region in the developing brain: Nkx6.1+ cells from the isthmus region and the rostral R1, Otx2+ cells from the caudal R1, and PAX7+ and Otp+ cells from the R1 alar plate (Aroca *et al*., 2006; Lorente-Cánovas *et al*., 2012). PAX7 seems to be expressed throughout the avian IPN, but *Otp* and *Nkx6.1* are expressed in the rostral parts of the IPN and *Otx2* in the caudal parts of the IPN (Lorente-Cánovas *et al*., 2012). The molecularly distinct cell types mentioned above have been shown to follow distinct and cell type-specific migration patterns (Lorente-Cánovas *et al*., 2012). Similar results to the avian IPN were demonstrated in Moreno-Bravo *et al*. (2014) study with mice which supports the evolutionary conserved development of the IPN among different species. After the embryonic development of the IPN, many projections to the IPN are formed during the postnatal development (Barr *et al*., 1987). Projections, such as cholinergic projections and substance P projections through FR and serotonergic projections from the raphe area, reach the IPN (Barr *et al*., 1987). These projections find their way to a particular set of regions in the IPN (Barr *et al*., 1987). The different subnuclei of IPN can be neuroanatomically determined at the time of birth, and the subnuclei reach their final size in rodents approximately one month after the birth (Barr *et al*., 1987). Results discussed above highlight the complexity of the IPN, in terms of development and neurotransmitter composition.

One of the more recent methods trying to reveal the complexity of the IPN and its connections has been the tracing experiments. In the study of Quina *et al*. (2017) adult mice were used to study the habenolopeduncular circuitry, and the authors were able to reveal connections from different IPN subnuclei to other regions in the brain (Quina *et al*., 2017). A set of molecular markers, such as choline acetyltransferase and substance P, were used to identify the cell types that receive or send projections of the IPN (Quina *et al*., 2017). With anterograde and retrograde tracing methods, Quina *et al*. (2017) could show that the ventral part of the MHb sends cholinergic projections to the rostral, dorsolateral, intermediate and the caudal subnucleus of the IPN, whereas, substance P projections from the dorsal MHb reach the lateral subnuclei of the IPN (Quina *et al*., 2017). On the other hand, IPN sends efferent projections to a various set of areas in the pons (Quina *et al*., 2017). Rostrolateral IPN sends projections to the dorsal tegmental nucleus alongside with the dorsolateral IPN, and the dorsolateral IPN, furthermore, send projections to the paramedian raphe nucleus (Quina *et al*., 2017). In addition, dorsomedial IPN sends its projections to the central grey of the pons, and the

median raphe nucleus and the lateral subnuclei of the IPN send their projections to the central grey of the pons and the nucleus incertus (Quina *et al*., 2017). Alongside with the complexity of projection patterns, IPN was divided into GABAergic and glutamatergic cells in the study of Quina *et al.* (2017). Their results showed that apart from rostrolateral subnuclei and parts of rostral subnucleus, which showed glutamatergic efferent projections, IPN is mainly a GABAergic nucleus (Quina *et al*., 2017).

Compared to the rodent IPN, the human IPN is cytoarchitectonically simpler, but still has a heterogeneous neurotransmitter pattern (Panigrahy *et al*., 1998). In the human IPN, three different compartments are defined in comparison to the eight separate compartments defined in rats (Groenewegen *et al*., 1986; Panigrahy *et al*., 1998). Through autoradiography studies of post-mortem samples of human foetuses and adults, different neurochemical expressions and localizations were mapped in the study of Panigraphy *et al*. (1998). Their results showed that the human IPN seemingly contains a set of active neurochemical receptors: muscarinic cholinergic receptors, opioid receptors and serotonergic receptors in all studied stages (Panigrahy *et al*., 1998). Furthermore, nicotinic cholinergic receptors and glutamatergic kainate receptors were expressed in the human IPN during development (Panigrahy *et al*., 1998). With these results from the autoradiography, the human IPN could be divided neurochemically into three different compartments: the medial with kainate and opioid receptors, lateral with muscarinic and nicotine acetylcholine receptors and dorsal subnuclei with serotonergic receptors (Panigrahy *et al.,* 1998).

Function of the Interpeduncular Nucleus

The main well-known functionality of the IPN is studied to relate to functions such as learning, male sexual behaviour, depression, addiction, anxiety and sleep (Hammer & Klinberg, 1990; Valjakka *et al*., 1998; Dermon *et al*., 1999; Funato *et al*., 2010; Padilla *et al*., 2011; Zhao-Shea *et al*., 2013; Zhao-Shea *et al*., 2015). The best-studied functions related to the IPN are the regulation of sleep and response to addiction, specifically nicotine addiction.

In the study by Haun *et al*. (1992), sleep patterns and especially the role of IPN in REM-sleep (Rapid eye movement sleep) was studied via cell transplantations and surgical lesions to the FR. This study was conducted by comparing FR-lesioned rats to wild type rats, as well as rats with cell transplantations in a behavioural test called the 'flower-pot-technique', which is designed to test REM-sleep atonia (Haun *et al*., 1992). The sleep studies of the MHb and the IPN (Haun *et al*., 1992)

were based on results from anaesthetized animals that had increased activity in these areas during anaesthesia (McQueen *et al*., 1984). In the Haun *et al*. (1992) study, rats with lesions in the FR showed reduced sleep episodes, especially in adult rats. The change was not as significant and closer to wild type rats with younger FR-lesion rats in the middle of their postnatal development (Haun *et al*., 1992). Cell transplants from either habenula or thalamus had the ability to restore the usual amount of sleep episodes in these FR-lesioned rats, both in adults and in younger rats (Haun *et al*., 1992). From these results, they interpreted that substance P projections relate to REM-sleep periods and cholinergic projections between MHb and IPN relate more to non-REM sleep in rodents depending on the implanted cell transplant (Haun *et al*., 1992). Moreover, they stated that FR and IPN seem to have more role in REM-sleep atonia than diminishing the whole REM-sleep completely due to visual interpretation of the condition of the animals that did not show REM-sleep deprivation (Haun *et al*., 1992). Relation of REM-sleep with the IPN has also been studied more recently by Funato *et al*. (2010) where the loss of Goosecoid homeobox 2 gene (*Gsc2*) in the IPN showed an altered pattern of REM-sleep in rats, which indicated a notable role for *Gsc2* expressing cells in the regulation of REM-sleep.

A study concerning the role of IPN in nicotine addiction has shown high expression of nicotinic acetylcholine α5-receptor (nAChRα5) in GABAergic cells of the rostral IPN that receive their projections from the MHb (Hsu *et al*., 2013). With immunohistochemistry, Hsu *et al*. (2013) located the nAChRα5 containing cells to the IPN instead of MHb which had been their hypothesis regarding the role of MHb in nicotinic addiction and especially in nicotinic withdrawal (Salas *et al*., 2009; Hsu *et al*., 2013). Overall, their results indicated that these GABAergic cells would presumably play a role in the behavioural aspects of nicotine, such as addiction and withdrawal (Hsu *et al*., 2013). In addition, Salas *et al.* (2009) showed that the lack of nAChRα5 and nAChRα2 in the IPN decrease the signs of nicotine withdrawal in mice substantially. It has also been demonstrated by Zhao-Shea *et al.* (2013) that the optical activation of GABAergic neurons in the IPN creates physically visible nicotine withdrawal symptoms in mice. Moreover, the effects of other addictive substances than nicotine, such as methamphetamine and cocaine, are shown to be related to the habenopeduncular network (Hussain *et al*., 2008).

Neuronal Cell Development in the Mammalian Midbrain and Hindbrain

The brainstem, a derivative of the midbrain and hindbrain and the focus in this study, originates – as does the whole central nervous system (CNS) – from the ectodermal layer, where the brain forms from the most anterior end of the folded ectoderm called the neural tube (reviewed in Ángeles Fernández-Gil *et al*., 2010). Forming of the neural tube is referred to as neurulation: a process where a sheet of ectodermic epithelial cells folds up into a tube-like form (Donkelaar *et al*., 2014).

Differentiation process from stem cells to neurons can be divided into patterning, neurogenesis alias post-mitotic exit from the cell differentiation and migration process (reviewed in Achim *et al*., 2014). The role of different transcription factors during this process has been studied by different methods and different species, and many transcription factors have shown to have different regulatory effects on different types of developing neurons (studied by, for example, Molyneaux *et al*., 2005; Holmberg *et al*., 2008; Kadkhodaei *et al*., 2009; Kala *et al*., 2009; Rouaux & Arlotta, 2010; Lorente-Cánovas *et al.*, 2012; Achim *et al*., 2013; Lahti *et al*., 2016). Especially a group of transcription factors, homeobox proteins, are known to be a key regulator neuronal development (reviewed in Vollmer & Clerc, 1998). Some of the transcription factors affecting during the neural development in the midbrain-hindbrain area have been characterized (Waite *et al*., 2012; Lahti *et al*., 2016), but many of them and roles of them are still under investigation.

The Development of Rhombomere 1 V2 Cell Types

New methods, such as single-cell RNA sequencing (scRNAseq), have abled neuroscientist to the particular mapping of cell populations and subpopulations in the nervous system (as demonstrated in, for example, Gokce *et al*., 2016; La Manno *et al*., 2016). The development of GABAergic interneurons in the midbrain-hindbrain area is characterized to a certain extent. For instance, it is known that GABAergic neurons in the substantia nigra pars reticulata (SNpr) that are related to the ventral midbrain dopaminergic nuclei originate from the R1 region of the hindbrain during mouse development (Achim *et al*., 2012). GABAergic neurons deriving from the R1 area are dependent on Tal1 (T-cell acute lymphocytic leukaemia protein 1) transcription factor expression (Lahti *et al*., 2016). Tal1 regulates expression of the transcription factors Gata2 and Gata3 (GATA-binding factor 2 and 3) that are both expressed in GABAergic neurons that are Gad1 (Glutamic acid decarboxylase) positive (Lahti *et al*., 2016). Furthermore, the loss of Tal1 expression increases the expression of

Nkx6.1+/Vsx2+ cells indicating that Tal1 has a role in neurotransmitter selection between GABA and glutamate during development in the R1 region (Lahti *et al*., 2016). GABAergic development in other brain regions is also quite intensively studied, especially the development of cortical interneurons regulating the glutamatergic pyramidal neurons in the cortex has been studied considerably (for example, by Anderson *et al*., 1997; Cauli *et al*., 1997; Butt *et al*., 2005; Fogarty *et al*., 2007; Azim *et al*., 2009; Paul *et al*., 2017).

In this study, the focus was on the development of a possible GABAergic neuron subpopulation derived from the R1 area: the *Gsc2* expressing neurons in the IPN. As mentioned previously, the cells of the IPN originate mainly from the R1 region and the isthmus region of the midbrain-hindbrain border (Lorente-Cánovas *et al*., 2012; Lahti *et al*., 2016). Gsc2 (also known as Goosecoid-like Gscl), is a transcription factor that is known to be expressed only in the IPN in the CNS, specifically in the caudal (IPc) and lateral (IPl) parts of the IPN (Gottlieb *et al*., 1998; Gong *et al*., 2003; Funato *et al*., 2010). Gsc2 was initially found in studies related to a developmental disorder called the DiGeorge syndrome that manifests, for example, as an abnormal neural crest cell development in humans (Gottlieb *et al*., 1997). *Gsc2* gene encodes for a homeodomain transcription factor, and it is expressed in the embryonic CNS starting from E9.5 (Gottlieb *et al*., 1998). *Gsc2* also has a downstream gene *Dgcr14* (DiGeorge Syndrome Critical Region 14), which is expressed similarly with *Gsc2* in the IPN but also in numerous other parts of the brain (Funato *et al*., 2010).

During development, the expression of *Gsc2* is highly specific to the pons area, from there *Gsc2* expressing (Gsc2+) cells migrate towards their final position in the IPN (Gottlieb *et al*., 1998; Gong *et al*., 2003; Funato *et al*., 2010; Ruiz-Reig *et al*., 2019). The tangential migration of Gsc2+ cells seems to be regulated by another caudal IPN homeobox gene *Otx2* (Orthodenticle homeobox 2) (Ruiz-Reig *et al*., 2019). It was initially shown that *Gsc2* knockout mice lack developmental abnormalities (Saint-Jore *et al*., 1998). However, Funato *et al*. (2010) found that *Gsc2* knockout mice experienced decreased time and a reduced number of REM-sleep periods, and reduced theta-power in EEG (Electroencephalography) during REM-sleep compared to wild type mice. What is still unknown about these Gsc2+ cells, is their cellular identity regarding their neurotransmitter component and what circuits they form with other nuclei in the brain. The main finding regarding this has been the co-localization with serotonin (5-HT) with Gsc2+ cells shown by Gottlieb *et al*. (1998) at E12.5 in mice, but those results have not been replicated by others.

Notch Signalling

Notch is a cell membrane receptor which is involved in many cell-to-cell interaction processes throughout the development (Wharton *et al*., 1985). The abnormalities in the neuronal differentiation caused by the lack of Notch in the nervous system development were discovered in *Drosophila melanogaster* (Poulson, 1937; Poulson, 1940), and hence Notch was defined as a neurogenic gene (Poulson, 1940; Lehmann *et al*., 1981). Notch signalling is activated via the binding of ligands, such as Delta or Serrate, to the Notch receptor's extracellular domain (Fehon *et al*., 1990; Rebay *et al*., 1991). The ligand binding induces the cleavage Notch intracellular domain (NICD) by presenilin dependent γ-secretase and NICD is transported to the nucleus to activate transcription of Notch target genes, such as *Hes* (Hairy and enhancer of split) transcription factors (Levitan & Greenwald, 1995; Levitan & Greenwald, 1998; Schroeter *et al*., 1998; Ray *et al*., 1999; De Strooper *et al*., 1999). Through Delta ligand binding into a Notch receptor in the neighbouring cell, Notch signalling cascade can be activated (Fehon *et al*., 1990). Delta-Notch interaction induces Notch target gene expression in the Notch expressing cells, while in the Delta expressing cells Delta expression becomes more prominent (Klein *et al*., 1997; Miller *et al*., 2009). The expression of Delta in neuronal progenitors promotes proneural gene expression, such as *Mash1* (Mammalian achaete-scute homolog-1), whereas the expression of Notch target genes, *Hes* transcription factors, promote the stemness of progenitors (reviewed in Kageyama *et al*., 2008). This way Notch signalling pathway has an essential role in neuronal developmental, for example, in lateral inhibition, gliogenesis and maintaining the pool of neuronal progenitors during neurogenesis (Showed by, for instance, Chenn & McConnell, 1995; Gaiano *et al*., 2000; Appel *et al*., 2001; Mizutani & Saito, 2005).

Notch Signalling in Cell Fate Selection

Notch signalling has a known role in nervous system development for determining is the cell kept in a stem cell status or is the cell moved further on to differentiate (Fortini *et al*., 1993; Nye *et al*., 1994). Whereas, the role of Notch as a determinant for neurotransmitter type in neurons is not as known. Some studies in invertebrates have linked Notch-signalling to cell fate decision; for example, Notch promoting the R4 cell fate in the retina of *Drosophila melanogaster* (Fanto & Mlodzik, 1999). In the vertebrate nervous system, the role of Notch in neurotransmitter acquisition has been shown in the spinal cord. Peng *et al*. (2007) showed that in the absence of Notch signalling, excitatory

interneurons (v2aIN) were produced in excess compared to inhibitory interneurons (v2bIN) in the p2 domain of the mouse and zebrafish spinal cord. Furthermore, the authors showed that the increase of Notch signalling increases the amount of v2bIN cells compared to v2aIN cells in the chick spinal cord (Peng *et al*., 2007). Peng *et al.* (2007) concluded that Delta4 – Notch1 signalling regulates the neurotransmitter identity, either GABA or glutamate, in the p2-domain of the spinal cord in vertebrates (Peng *et al*., 2007). These results lead to a question, could Delta-Notch signalling have a similar role in determining the neurotransmitter identity of neurons also in other parts of the CNS.

Aims of the Study

The aim for this master's thesis project was to validate lineage-specific genes, predicted by scRNAseq performed on embryonic mice at the age of E13.5 and E15.5 (Morello *et al*., unpublished; Fig. 1), as well as to analyse the co-expression and mutually exclusive expression of the lineage markers. The validation process was performed with embryonic mice at the age of E12.5 and E15.5 using in situ hybridisation (ISH) and immunohistochemistry (IHC). The focus in this study was on GABAergic neuronal subpopulations and how they are established during development in the rhombencephalic ventral 2 (rV2) domain of the R1 region in the hindbrain. The neurotransmitter selection and development of subpopulation of GABAergic cells of the IPN were assessed in this study.

This background taken into consideration the research questions for this master's thesis were:

1. When and where is *Gsc2* expressed in the embryonic mouse CNS, and what is the cell type of *Gsc2* expressing cells?
2. Is *Gsc2* co-expressed with *Sall3* (Spalt like transcription factor 3) in the rV2 domain of R1 or the interpeduncular nucleus during development?
3. Does the inactivation of Notch signalling affect in the GABAergic versus glutamatergic fate selection in the rV2 domain in the developing mouse brain?

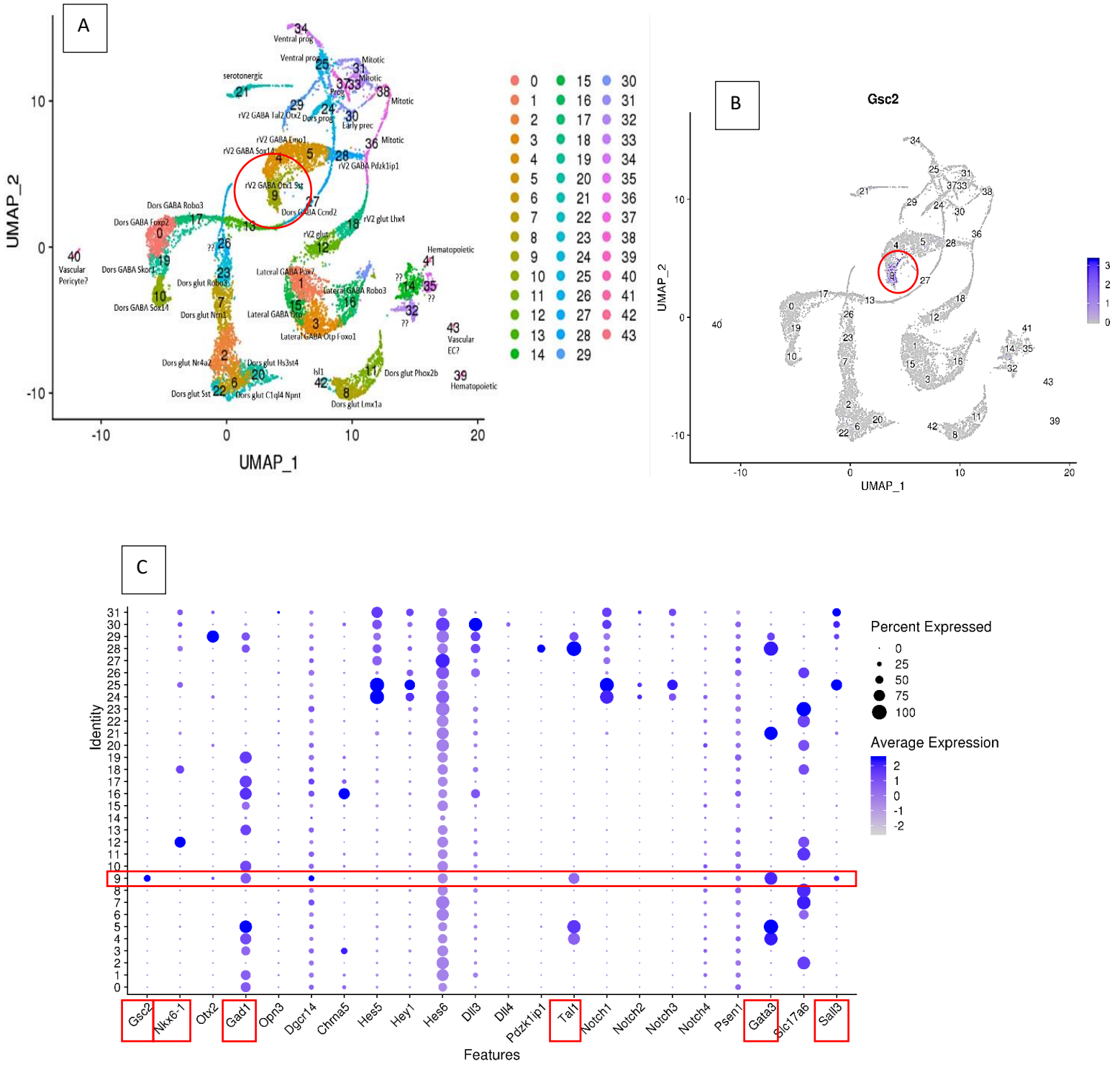

Figure 1

Clustering of E13.5 mice. A: UMAP showing the clustering of R1 cells. Cluster 9 highlighted with a red circle represents the rV2 region, which gives rise to GABAergic cells. B: UMAP showing the expression of *Gsc2* in cluster 9. C: A dot plot showing a set of genes and their expression magnitude in different clusters. Y-axis: cluster numbers. X-axis: different markers. Cluster 9 is highlighted with a red box and the markers used in this study are highlighted with small red boxes (*Gsc2*, Nkx6.1, *Gad1, Tal1, Gata3* and *Sall3*). Single-cell RNA sequencing data was collected by Kaia Achim. Figures and clustering were done by Samir Sadik-Ogli with R package Seurat (Butler *et al*., 2018).

Materials and Methods

Mice

In this study, mouse embryos at the age of E12.5 and E15.5 were used. For the analysis of gene expression in GABAergic cells, including the expression of *Gsc2,* Gad67 $^{egfp/wt}$ mouse strain was used. The Gad67 ^{egfp} strain, where the *EGFP* gene is expressed from the Gad67 locus, was obtained from Tamamaki laboratory (Tamamaki *et al*., 2003) and was maintained in an ICR-background in the mouse facility of University of Helsinki. Gad67 $^{egfp/wt}$ mouse can be used to visualise the expression of Gad67 (Glutamic acid decarboxylase 67) with a green fluorescent protein (GFP) that could be interpreted as GABAergic cells. Glutamic acid decarboxylase functions as an enzyme catalysing glutamate to turn into GABA (Wu *et al*., 2018). For the Notch signalling related experiments, a heterozygous presenilin 1 ($Psen1^{+/-}$) mice were mated. The $Psen1^{-/-}$ strain, where the knockout of presenilin 1 gene was performed using homologous recombination on exon 7, was obtained as embryos from the European Mouse Mutant Archive (De Strooper *et al*., 1998) by Juha Partanen and it was maintained in the mouse facility of University of Helsinki. $Psen1^{-/-}$ embryonic mice were used with wild type controls from the same litter.

All the used embryos were obtained at the timed matings of the mouse strains used. Since the experiments were conducted using embryonic mice, the sex was not determined for specific individual embryos. All the experiments were repeated with several embryos from the same litter for each stage. The plugging and maintenance of animals were conducted by the mice facility of the University of Helsinki. At the time of euthanasia, every effort was made to minimise the produced discomfort or pain of the mice. All the experiments conducted with these mice were approved by the National Animal Experiment Board in Finland and by the Laboratory Animal Centre, University of Helsinki.

Collection of Embryos and Tissue Processing

To confirm the stage of the embryonic animals, the vaginal plug day was marked as E0.5. The embryos (E12.5) or embryonic brains (E15.5) were dissected, and Gad67-GFP positive embryos were separated from the wild type embryos under a fluorescent microscope (Leica MZ FL III). $Psen1^{-/-}$

embryos were identified by genotyping using polymerase chain reaction (PCR) by Juha Partanen laboratory. Primer sequences used for genotyping the Psen1$^{-/-}$ mice were: Psen1-wt-Forward: GTA GGG GAT ATG ATT TTC TTT TTG; Psen1-wt-Reverse: CCA TTC GGG GAG GTA CTT GAT AA; Psen1$^{-/-}$-Forward: CGG ATC AGG CGT ATG CAG CCG; Psen1$^{-/-}$-Reverse: CAT ATA CTG AAA TCA CAG CCA AG. Embryos or embryonic brains were fixed two to five days in 4% paraformaldehyde (PFA; Sigma-Aldrich, Cat#P6148) in PBS (Phosphate-buffered saline) in room temperature. After the fixation, samples were dehydrated overnight and embedded in paraffin (Merck). After the paraffin embedding, embryos or embryonic brains were sectioned in coronal orientation, sagittal orientation, transverse orientation or to cross-sections with a microtome (Leica RM2255) to 5µm thick parallel paraffin sections from the midbrain and hindbrain area according to the Allen Developing Mouse Brain Atlas (2008). Finally, the sections were collected to adjacent microscope slides.

Immunohistochemistry

Immunohistochemistry was used in this study to visualise the expression of wanted proteins in collected tissue sections. First, the suitable paraffin sections were rehydrated using series from xylene to alcohol to water in a stepwise manner. Next, the sections were permeabilised for 45 minutes with 0.3% Triton X-100 (Sigma-Aldrich, Cat#T9284) in PBS and the antigen retrieval was performed boiling the sections in the microwave oven in 0.1M sodium citrate buffer (pH 6) for 13 minutes. After the antigen retrieval, the sections were blocked using 10% goat serum, 1% BSA and 0.3% Triton X-100 in PBS solutions for 1 hour. The incubation with the primary antibodies diluted in 1%BSA and 0.3% Triton X-100 in PBS solution was performed after the blocking. Incubation time for the primary antibodies was 48 to 72 hours at +4˚C. After the primary antibody incubation, the secondary antibody was added to the sections, incubated for 4 hours in room temperature, and counter-stained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, Cat#D9564) for visualizing the nuclei of cells. Finally, the sections were mounted with FluorSave reagent (Merck, Cat# 345789-20ML). Dilutions of the primary antibodies used in this study: rabbit anti-GFP (1:1000, Abcam, ab290); mouse anti-Nkx6.1 (1:1000, from Madsen, Ole D., F55A10, (Pedersen *et al*., 2006)); mouse anti-Gata3 (1:200, Santa Cruz Biotechnology, sc-268); rabbit anti-5-HT (1:500, Immunostar, Cat#20080). The secondary antibodies used in this study (1:400): donkey anti-rabbit IgG Alexa Fluor

488 (Life Technologies, Cat#A21206); donkey anti-mouse IgG Alexa Fluor 488 (Life Technologies, Cat#A21202).

In Situ mRNA Hybridisation

In situ hybridisation was used in this study to visualise the mRNA (messenger ribonucleic acid) expression in selected tissue sections. Digoxigenin- or fluorescein-labelled cRNA probes were used, and the probes were synthesised with specific RNA labelling kits (Roche, Cat#11277073910 and Cat#11685619910 respectively) following the recommendations by the manufacturer. cRNA in situ probes used in this study: Gsc2 (Source BioScience, IRCLp5011G0810D, IMAGE40129871); Sall3 (RP_051121_01_B02 from Allen Brain Atlas (Lein *et al*., 2007)); Tal1 (Source BioScience, IRAVp968D09118D); Gad1 (RZPD, IRAVp968C1167D, IMAGE5358787).

The selected paraffin sections were rehydrated using series from xylene to alcohol to water in a stepwise manner. Next, the sections were permeabilised for 10 minutes with 0.3% Triton X-100 in PBS and the antigen retrieval was performed boiling the sections in the microwave oven in 0.1M sodium citrate buffer (pH 6) for 13 minutes. Additional permeabilisation was performed on sections using 20% SDS (Sodium dodecyl sulphate) in PBS for 15 minutes and treated with 0.25% acetic anhydride (Sigma-Aldrich, Cat#33214) in 0.1M TEA (Triethanolamine; Sigma-Aldrich, Cat#33729) for 10 minutes on a shaking platform. Then, graded ethanol series was used to dehydrate the sections. After the dehydration, sections were left to dry in room temperature for at least an hour. Meanwhile, the probes were diluted (depending on probe to gain ~1ng concentration) in hybridisation buffer (10% Dextran sulphate (Sigma-Aldrich, Cat#D8906), 0.3M NaCl, 20mM Tris-HCl (pH 8.0), 5mM EDTA (pH 8.0), 1xDenhardt's solution (Sigma-Aldrich, Cat#D2532), 50% Ultrapure formamide (Invitrogen, Cat#15515-026), 500ug/ml Yeast RNA (Sigma, Cat#R6750)) and added to the slides. An overnight incubation at +65˚C was performed on slides for the hybridisation to occur.

The following day slides were washed with 5xSSC (Saline sodium citrate) and subsequently with 50% formamide (Millipore, Cat#75-12-7) in 2xSSC in a water bath at +65˚C for 30 minutes to 1 hour. After the washes, sections were treated with RNAseA (0,02mg/ml, Roche, Cat#10109169001) in NTE (0.5M NaCl, 5mM Tris-HCl pH8.0, 5mM EDTA pH8.0) at +37˚C for 45 minutes. Next, the blocking of the sections was performed using TNB blocking buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent (Perkin Elmer, Cat#FP1012)) for 1 hour and incubated with sheep anti-Digoxigenin-

POD (Peroxidase) Fab fragments antibody (Roche, Cat#11207733910, 1:1000) or sheep anti-Fluorescein-POD, Fab fragments (Roche, Cat#11426346910, 1:800) antibody at +4˚C overnight. For the detection of incubated antibody, the TSA Plus Cyanine 3.5 (PerkinElmer, Cat#NEL763B001KT) was used, giving the sections a red fluorescent signal (RFP). Slides were then counter-stained with DAPI. Finally, slides were mounted with FluorSave Reagent (Merck). To combine in situ hybridisation with immunohistochemistry, the normal immunohistochemistry was performed on the slides starting from the blocking step.

Image Analysis

Images from the sections were taken with an Olympus BX63 microscope with the DP72 camera and further processed with Fiji ImageJ and Microsoft Office software package. Brightness, contrast and sharpness of the pictures were adjusted to demonstrate the original tissue samples seen from the microscope.

Results

Gsc2 Expressing Cells Originate from the R1 Region

The focus of this study was in a small nucleus in the midbrain-hindbrain boundary of the mammalian brain, the interpeduncular nucleus. Particularly, a homeobox gene Goosecoid homeobox 2 was of interest. The gene was chosen due to its specific expression only in the IPN and not in the other parts of the brain (Gottlieb *et al*., 1998; Gong *et al*., 2003; Funato *et al*., 2010). From scRNAseq results, these cells expressing *Gsc2* are GABAergic showing a co-expression pattern with GABAergic markers, such as Gad1 and Gata3 (Fig. 1C), and could be therefore a subpopulation of GABAergic neurons in the IPN. Also, according to the scRNAseq results, a population of Gsc2*+* cells would originate from the rV2 domain of the R1 region (Fig. 1A-B). We used in situ hybridisation to detect *Gsc2* mRNA expression during mouse brain development. At E12.5, the expression of *Gsc2* can be seen alongside more posterior part of the R1 region creating a line across the dorsal-ventral axis of the hindbrain (Fig. 2A' & Fig. 3B') consistent with the results gained by others (Gottlieb *et al*., 1998; Saint-Jore *et al*., 1998; Gong *et al*., 2003; Funato *et al*., 2010). As predicted from the scRNAseq

results (Fig. 1A-B), Gsc2+ cell population originates from the rV2 domain, supported by the results gained by Gottlieb *et al*. (1998). Our results show that there might be a small population of Gsc2+ cells originating from the rV2 domain, which can be seen from the cross-sections of the R1 area (Fig. 4A'). However, the population in the rV2 is much smaller compared to the total number of Gsc2+ cells in the R1. At the age of E15.5, many Gsc2+ cells seem to have migrated close to their final position in the IPN (Fig. 5A' & Fig. 6A'). Some cells, however, can be still found from the posterior R1 region (non-migratory cells, Fig. 6A'). This shows that the IPN cell migration is a slow process extending many days during embryonic development and postnatal development (as indicated by Gottlieb *et al*., 1998 and Gong *et al*., 2003).

Gsc2 Expressing Cells are GABAergic

The neurotransmitter identity of these Gsc2+ cells was studied using combined immunohistochemistry and in situ hybridisation on the same slides, or by comparing the expression of relevant markers on adjacent sections in separate slides. Co-staining was performed on Gad67 $^{egfp/wt}$ transgenic embryos, where GFP is expressed in the locus of Gad67 and this way GABAergic neurons are labelled with GFP. Furthermore, separate immunohistochemistry analysis was performed on E15.5 wild type embryonic mice, using Gata3 as another GABAergic marker. At the age of E12.5, Gad67 is co-expressed with *Gsc2*, demonstrating that the Gsc2+ cells are GABAergic (Fig. 2 & Fig. 3). We also detected the expression of *Gad1* mRNA in the region of *Gsc2* expressing cells at the age of E12.5 mice in the rV2 domain (Fig. 4B'). At the age of E15.5, expression of GABAergic markers Gad67 or Gata3 with Gsc2+ cells is not as distinguishable as at E12.5 (Fig. 5 & Fig. 6). In transverse sections of E15.5 mice, the expression of Gad67 is visible in the Gsc2+ migrating cells, but not so much in the Gsc2+ cells already located in the IPN region (Fig. 6B''' & Fig. 6C'''). Moreover, Gad67 co-expression with *Gsc2* in coronal sections at E15.5 is slightly fainter than at E12.5 but still visible (Fig. 2 & Fig. 5). To highlight the GABAergic identity of Gsc2+ cells, co-expression with the serotonergic marker, 5-HT, was not evident in our results at E12.5 (Fig. 4C') although it has been shown previously that *Gsc2* would have co-expression with 5-HT (Gottlieb *et al*., 1998).

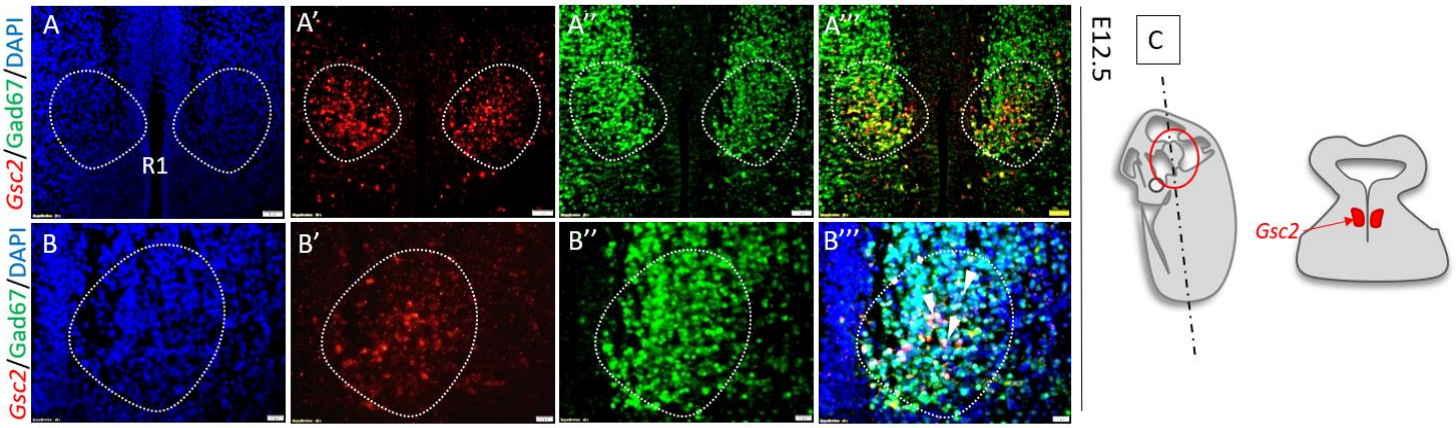

Figure 2

Analysis of the expression of *Gsc2* and Gad67 was studied on coronal sections of E12.5 R1 region. A-A''': Cell nuclei visualised by DAPI (A). Analysis of the expression of *Gsc2* (A', ISH) and Gad67 (A'', IHC). Merge of the *Gsc2* and Gad67 stains showing the co-expression of the markers in yellow (A'''). Scale bar: 50µm. B-B''': Cell nuclei visualised by DAPI (B). Analysis of the DAPI (B), of *Gsc2* (B', ISH) and Gad67 (B'', IHC). Co-expression of *Gsc2* and Gad67 presented in a merged picture with DAPI (B'''). White arrows are pointing to the cells co-expressing *Gsc2* and Gad67. Scale bar: 20µm. C: Schematic representation of the orientation and *Gsc2* expression in E12.5 mouse brain.

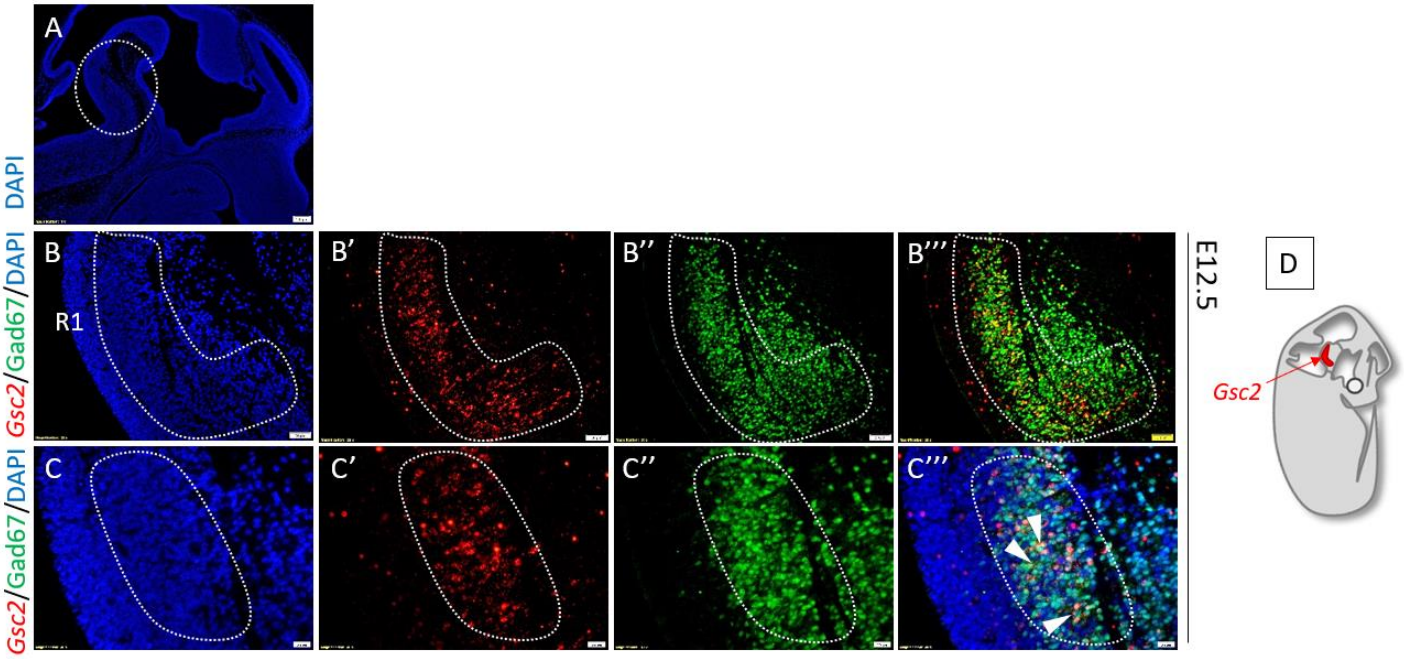

Figure 3

Analysis of the expression of *Gsc2* and Gad67 was studied on sagittal sections of E12.5 R1 region. A: Cell nuclei visualised by DAPI. Scale bar: 200µm. B-B''': Cell nuclei visualised by DAPI (B). Analysis of the expression of *Gsc2* (B', ISH) and Gad67 (B'', IHC). Merge of the *Gsc2* and Gad67 stains showing the co-expression of the markers in yellow (B'''). Scale bar: 50µm. C-C''': Cell nuclei visualised by DAPI (C). Analysis of the DAPI (C), of *Gsc2* (C', ISH) and Gad67 (C'', IHC). Co-expression of *Gsc2* and Gad67 presented in a merged picture with DAPI (C'''). White arrows are pointing to the cells co-expressing *Gsc2* and Gad67. Scale bar: 20µm. D: Schematic representation of the orientation and *Gsc2* expression in E12.5 mouse brain.

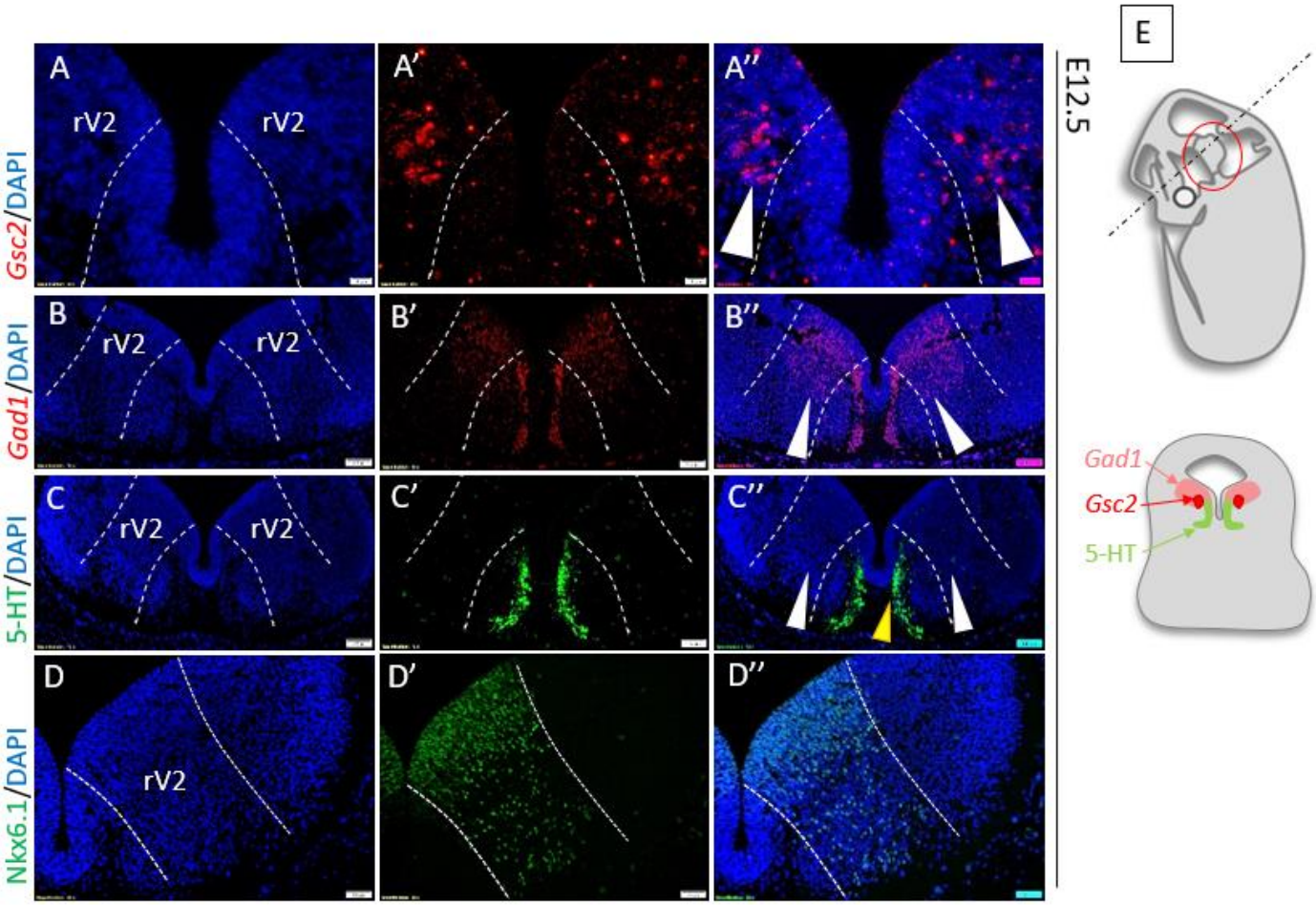

Figure 4

Analysis of the expression of *Gsc2*, *Gad1*, 5-HT and Nkx6.1 was studied on cross-sections of R1 area in E12.5 rV2 domain. A-A'': Cell nuclei visualised by DAPI (A). Analysis of the expression of *Gsc2* (A', ISH). White arrows are demonstrating *Gsc2* expression in a merged picture with DAPI (A''). Scale bar: 20µm. B-B'': Cell nuclei visualised by DAPI (B). Analysis of the expression of *Gad1* (B', ISH). White arrows are demonstrating *Gad1* expression in a merged picture with DAPI (B''). Scale bar: 50µm. C-C'': Cell nuclei visualised by DAPI (C). Analysis of the expression of 5-HT (C', IHC). White arrows are demonstrating the lack of 5-HT expression in a merged picture with DAPI (C''). A yellow arrow is demonstrating the location of the expression of 5-HT (C''). Scale bar: 50µm. D-D'': Cell nuclei visualised by DAPI (D). Analysis of the expression of Nkx6.1 (D', IHC). Nkx6.1 expression demonstrates the borders of the rV2 domain in a merged picture with DAPI (D''). Scale bar: 50µm. E: Schematic representation of the orientation and expression of *Gsc2*, *Gad1* and 5-HT in E12.5 mouse brain.

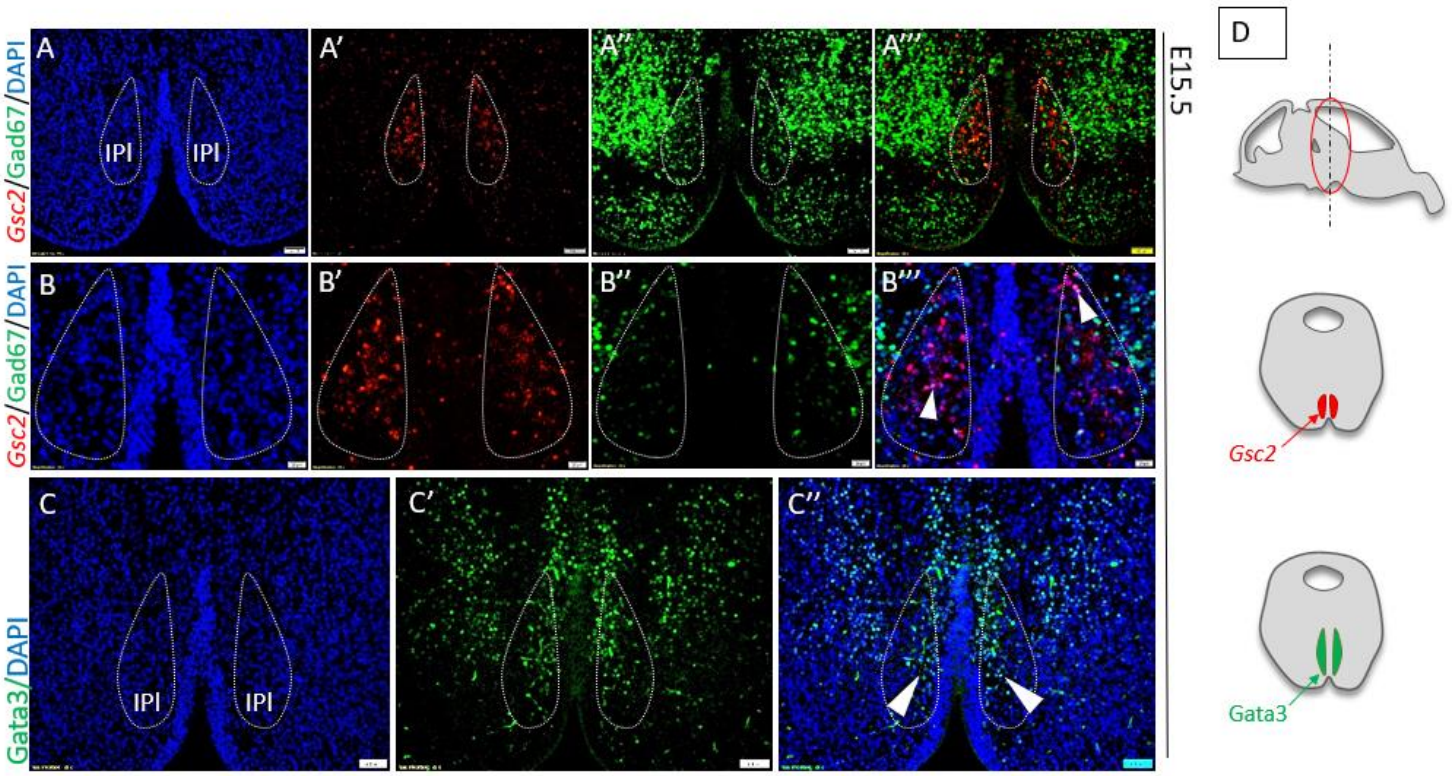

Figure 5

Analysis of the expression of *Gsc2*, Gad67 and Gata3 was studied on coronal sections of E15.5 IPl region. A-A''': Cell nuclei visualised by DAPI (A). Analysis of the expression of *Gsc2* (A', ISH) and Gad67 (A'', IHC). Merge of the *Gsc2* and Gad67 stains showing the co-expression of the markers in yellow (A'''). Scale bar: 50µm. B-B''': Cell nuclei visualised by DAPI (B). Analysis of the DAPI (B), of *Gsc2* (B', ISH) and Gad67 (B'', IHC). Co-expression of *Gsc2* and Gad67 presented in a merged picture with DAPI (B'''). White arrows are pointing to the cells co-expressing *Gsc2* and Gad67. Scale bar: 20µm. C-C'': Cell nuclei visualised by DAPI (C). Analysis of the expression of Gata3 (C', IHC). White arrows are demonstrating Gata3 expression in a merged picture with DAPI (C''). Scale bar: 50µm. D: Schematic representation of the orientation, and the expression of *Gsc2* and Gata3 in E15.5 mouse brain.

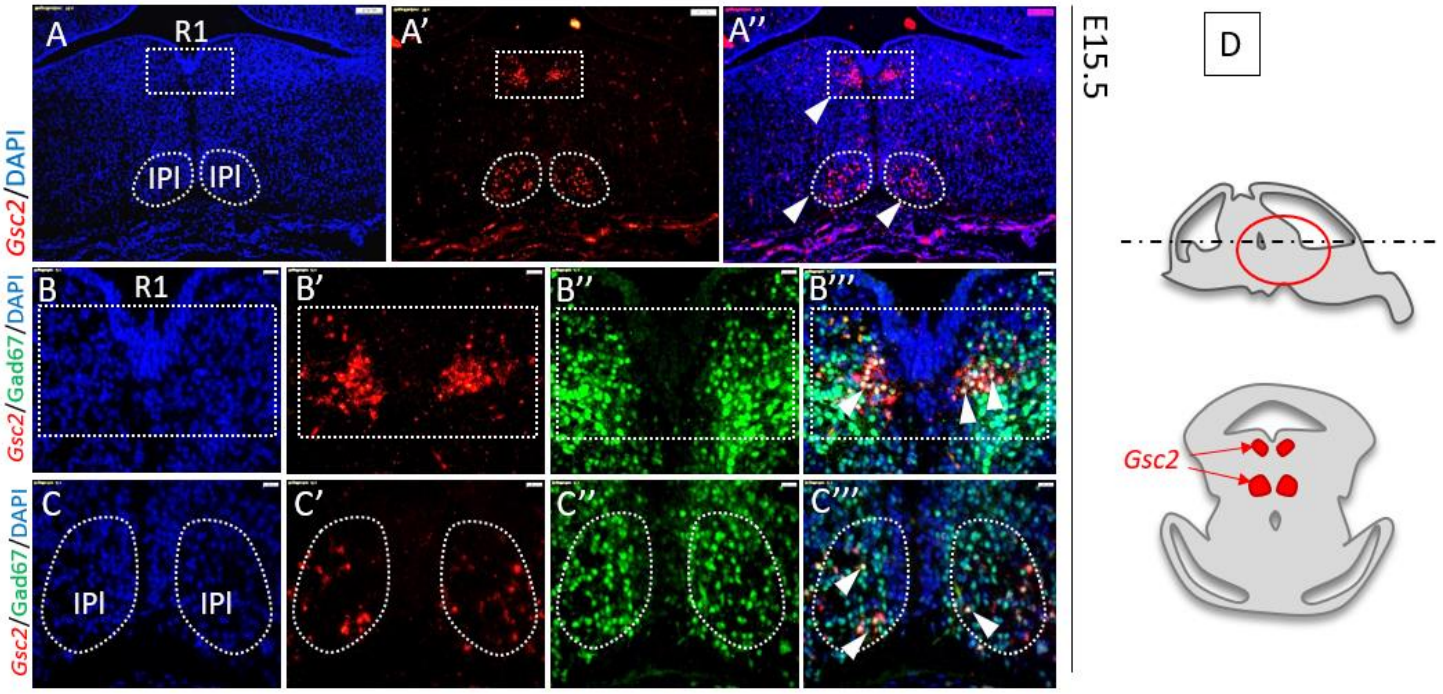

Figure 6

Analysis of the expression of *Gsc2* and Gad67 was studied on transverse sections of E15.5 R1 and IPl region. A-A'': Cell nuclei visualised by DAPI (A). Analysis of the expression of *Gsc2* (A', ISH). White arrows are demonstrating *Gsc2* expression in a merged picture with DAPI (A''). Scale bar: 100µm. B-B''': Cell nuclei visualised by DAPI (B). Analysis of the DAPI (B), of *Gsc2* (B', ISH) and Gad67 (B'', IHC). Co-expression of *Gsc2* and Gad67 presented in a merged picture with DAPI (B'''). White arrows are pointing to the cells co-expressing *Gsc2* and Gad67. Scale bar: 20µm. C-C''': Cell nuclei visualised by DAPI (C). Analysis of the DAPI (C), of *Gsc2* (C', ISH) and Gad67 (C'', IHC). Co-expression of *Gsc2* and Gad67 presented in a merged picture with DAPI (C'''). White arrows are pointing to the cells co-expressing *Gsc2* and Gad67. Scale bar: 20µm. D: Schematic representation of the orientation and expression of *Gsc2* in E15.5 mouse brain.

Gsc2 is not Co-expressed with Transcription Factor Sall3

Predicted by the scRNAseq results (Fig. 1A-B), Gsc2+ cells are born in the rV2 lineage, where a variety of molecularly distinct progenitors are located (Morello *et al*., unpublished). One of those is Sall3+ progenitors, which are found in a similar position with Gsc2+ progenitors. *Sall3* is shown to be expressed in the serotonergic neurons in the raphe area of the mammalian brain (Morello *et al*., unpublished) and due to the near location of raphe and the IPN in the brain, the co-expression during development with *Gsc2* could be considered. To understand if *Gsc2* and *Sall3* lineages are partially or entirely related to one another, we decided to map the co-expression of *Gsc2* and *Sall3* at the age E12.5 and E15.5 of mice. Our in situ hybridisation results show that while both groups of cells expressing either one of these two markers are born in the R1 region, they are born in different sub-regions of that area. At E12.5, *Sall3* expressing cells seemingly originate from the rV2 domain, whereas the *Gsc2* expressing cells originate mainly from the more posterior part of the R1 (Fig. 3B'; Fig. 7A; Fig. 7B). The small cell population of Gsc2+ cells that was found from the cross-sections of R1 in the rV2 domain at E12.5 (Fig. 4A') is still located more laterally compared to the *Sall3* expressing cells (Fig. 7B''). At the age of E15.5, co-expression of *Sall3* and *Gsc2* is not seen in the in situ hybridisation results as could be predicted from the gained E12.5 results (Fig. 7C & Fig. 7D).

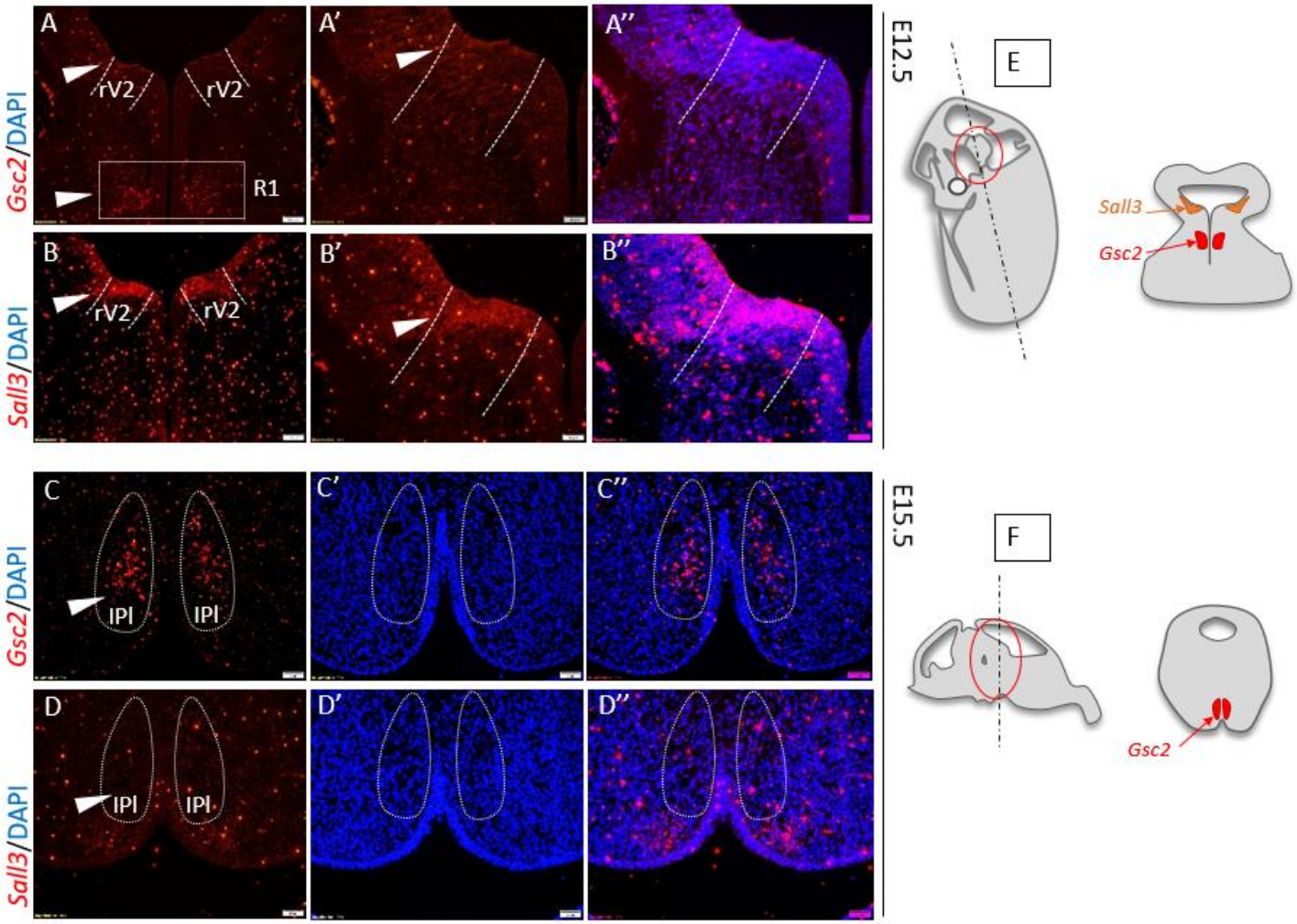

Figure 7

Analysis of the expression of *Gsc2* and *Sall3* was studied on coronal sections of E12.5 R1 region and E15.5 IPl region. A-A'': Cell nuclei visualised by DAPI (A''). Analysis of the expression of *Gsc2* (A-A', ISH). White arrows are demonstrating *Gsc2* expression in the R1 region and the lack of *Gsc2* expression in the rV2 domain (A-A'). Merge of the *Gsc2* and DAPI stains showing the lack of expression of *Gsc2* in the rV2 domain (A''). Scale bars: 100µm (A) and 50µm (A'-A''). B-B'': Cell nuclei visualised by DAPI (B''). Analysis of the expression of *Sall3* (B-B', ISH). White arrows are demonstrating *Sall3* expression in the rV2 domain (B-B'). Merge of the *Sall3* and DAPI stains showing the expression of *Sall3* in the rV2 domain (B''). Scale bars: 100µm (B) and 50µm (B'-B''). C-C'': Cell nuclei visualised by DAPI (C'). Analysis of the expression of *Gsc2* (C, ISH). The white arrow is demonstrating *Gsc2* expression in the IPl region (C). Merge of the *Gsc2* and DAPI stains showing the expression of *Gsc2* in the IPl region (C''). Scale bar: 50µm. D-D'': Cell nuclei visualised by DAPI (D'). Analysis of the expression of *Sall3* (D, ISH). The white arrow is demonstrating the lack of *Sall3* expression in the IPl region (D). Merge of the *Sall3* and DAPI stains showing the lack of expression of *Sall3* in the IPl region (D''). Scale bar: 50µm. E: Schematic representation of the orientation and expression of *Gsc2* and *Sall3* in E12.5 mouse brain. F: Schematic representation of the orientation and expression of *Gsc2* in E15.5 mouse brain.

Notch-Signalling Plays a Role in GABAergic Cell Fate Selection

Developmentally it is still not entirely clear how the cell fate is determined, for example, between GABAergic and glutamatergic cell fates. Our results showed that Gsc2+ expressing cells are GABAergic, and to show how GABA neurotransmitter identity is determined during development, the cell fate specification of neurons was assessed in this study. Notch-Delta-signalling has been implicated in cell fate switch in other areas of the CNS, such as the spinal cord (Peng *et al*., 2007). The area where these cells arise from is similar to the rV2 area in the R1 where many of the IPN neurons are derived from (Lahti *et al*., 2016; Morello *et al*., unpublished). Therefore, it might be that in this rV2 domain, Notch-Delta-signalling could also work as a determinant for cell fate specification. In addition, predicted by the scRNAseq-results, Notch protein would play a role in fate selection between glutamate and GABA as a neurotransmitter of neurons during midbrain-hindbrain development (Morello *et al*., unpublished). The scRNAseq-results showed that at a certain time point of development neurons divide into two lineages, a GABAergic lineage and to glutamatergic lineage (Morello *et al*., unpublished). The point where neurons fall into either of those categories, high expression either of Notch or its ligand Delta could determine what is the neurotransmitter type of that specific neuron (Morello *et al*., unpublished). A higher expression of Notch would indicate a GABAergic fate and higher expression of Delta would indicate a glutamatergic fate for that neuron in the rV2 domain (Morello *et al*., unpublished). To address this question, Psen1$^{-/-}$ embryonic mice at the age of E12.5 were used in comparison to wild type littermate controls to determine the effect of Notch-signalling in the rV2 domain.

From a set of markers predicted from the scRNAseq results (Morello *et al*., unpublished), GABAergic markers Gata3, *Gad1* and *Tal1* were used in this study to see the expression and possible change of GABA between Psen1$^{-/-}$ embryos and wild type embryos. To distinguish cells expressing or the difference of the expression of glutamate between transgenic and wild type embryos, *Vglut2* (Vesicular glutamate transporter) probe and Vsx2 (Visual system homeobox 2) antibody were tried to be used in this study. Due to technical difficulties with the glutamatergic markers (weak signal or methodological issues), none of the used glutamatergic markers functioned. Therefore, results from this study are based on the used GABAergic markers only. Notch-signalling was studied primarily in the rV2 domain, related to studies with *Gsc2* expressing cells, and a known marker for the region, Nkx6.1 antibody, was used to mark the borders of the rV2 domain (Fig. 8J; Fig. 8K; Waite *et al*., 2012; Lahti *et al*., 2016).

The in situ hybridisation and immunohistochemistry results show that the expression of used GABAergic markers decreases between the wild type embryos and Psen1$^{-/-}$ embryos (Fig. 8A; Fig. 8D; Fig. 8G, and Fig. 8B; Fig. 8E; Fig. 8H, respectively). The decrease in expression of the probe *Tal1* between the wild type embryo (Fig. 8A) and the Psen1$^{-/-}$ embryo (Fig. 8B) is evident in the mantle zone where there is less or almost none of the RFP signal left in the Psen1$^{-/-}$ rV2 domain compared to the wild type. Moreover, decrease in the antibody Gata3 is seen between the wild type embryo (Fig. 8G) and the Psen1$^{-/-}$ embryo (Fig. 8H) with fewer cells expressing Gata3 in the rV2 domain in the transgenic mouse embryo. The decrease is less evident in the reduction of *Gad1* probe signal between the compared subjects (Fig. 8D & Fig. 8E). Nearby the ventricular zone there can be seen few areas with less RFP signal from *Gad1* in the Psen1$^{-/-}$ embryo (Fig. 8E), but overall the RFP signal between the wild type embryo (Fig. 8D) and the Psen1$^{-/-}$ embryo (Fig. 8E) are spread quite evenly. In conclusion, inhibition of Notch signalling at the time point E12.5 during mouse development also decreases the expression of different GABAergic markers in the rV2 domain of R1, but it does not diminish the expression of these GABAergic markers completely.

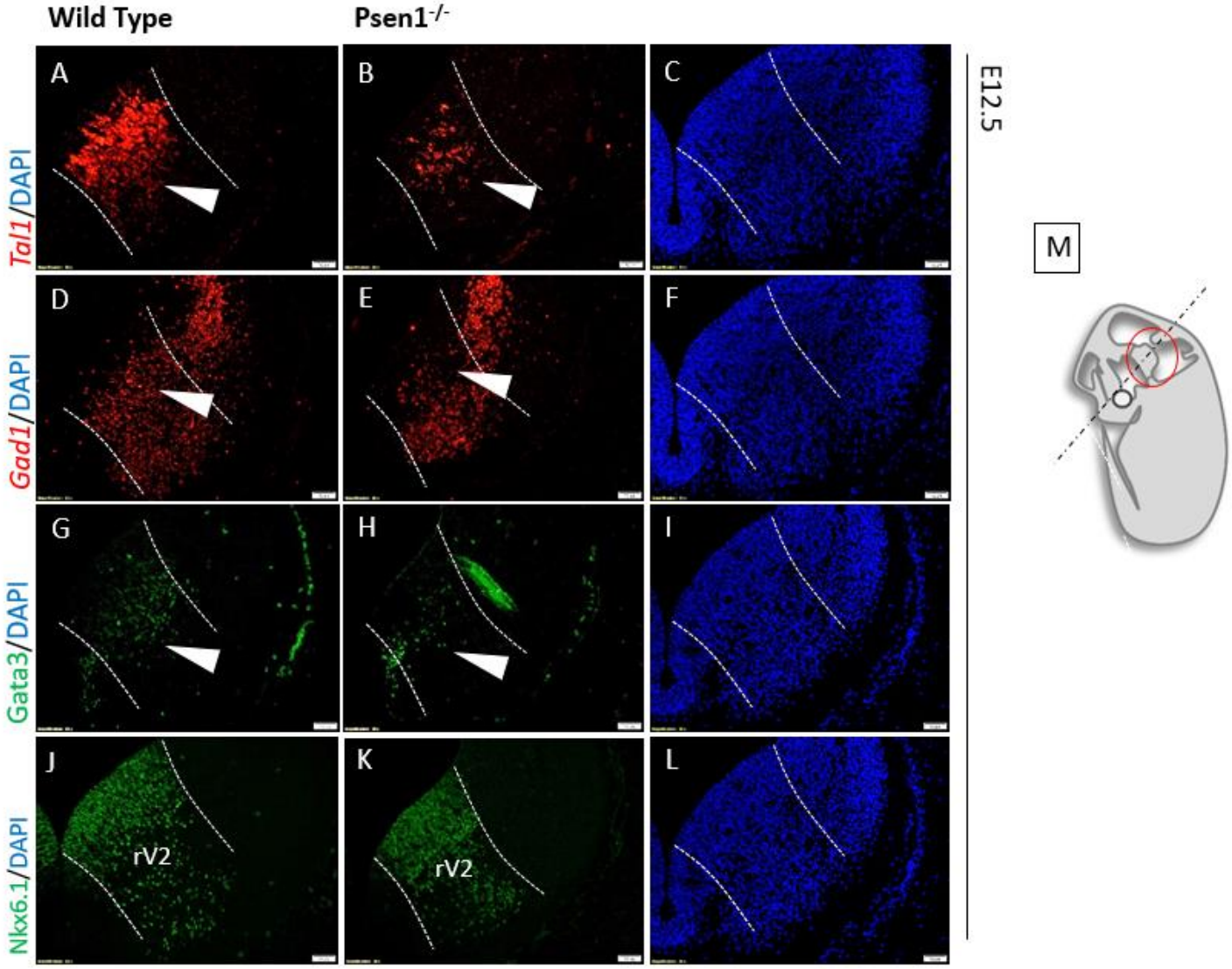

Figure 8

Analysis of the expression of *Tal1*, *Gad1* and Gata3 was studied on cross-sections of R1 area in wild type and Psen1$^{-/-}$ E12.5 rV2 domain. A-C: Cell nuclei visualised by DAPI (C). Analysis of the expression of *Tal1* (A, B, ISH). White arrows are demonstrating *Tal1* expression in the wild type (A) and Psen1$^{-/-}$ (B) mice. Scale bar: 50µm. D-F: Cell nuclei visualised by DAPI (F). Analysis of the expression of *Gad1* (D, E, ISH). White arrows are demonstrating *Gad1* expression in the wild type (D) and Psen1$^{-/-}$ (E) mice. Scale bar: 50µm. G-I: Cell nuclei visualised by DAPI (I). Analysis of the expression of Gata3 (G, H, IHC). White arrows are demonstrating Gata3 expression in the wild type (G) and Psen1$^{-/-}$ (H) mice. Scale bar: 50µm. J-L: Cell nuclei visualised by DAPI (L). Analysis of the expression of Nkx6.1 (J, K, IHC). Nkx6.1 expression demonstrates the borders of the rV2 domain (J, K). Scale bar: 50µm. M: Schematic representation of the orientation in E12.5 mouse brain.

Discussion

Gsc2 is a Potential Lineage Marker for GABAergic Cells in the IPN

Studies over the years have extensively mapped and characterised the cellular diversity of GABAergic cell types in the developing mouse midbrain-hindbrain region. Our results contribute to this characterisation by confirming the origin and neurotransmitter identity of a cell population located in the IPN. A group of cells expressing transcription factor Gsc2 originate from the R1 region and migrate from there to its final position in the IPc and IPl (as shown before by Gottlieb *et al*., 1998; Saint-Jore *et al*., 1998; Gong *et al*., 2003; Funato *et al*., 2010). Furthermore, our results show that these cells are co-expressing a GABAergic marker, Gad67, and thus are GABAergic. Some of the Gad67 expression decreased over time between E12.5 and E15.5 but did not disappear completely, which demonstrates the GABAergic nature of these cells. These cells are most likely interneurons that use GABA as their neurotransmitter. In situ hybridisation was used to identify the expression of *Gsc2*, and to test the GABAergic nature of these cells, immunohistochemistry was used to show the production of Gad67 protein. Furthermore, the in situ hybridisation analysis also shows the expression of R1 GABAergic neuron marker Gata3 in the same area than the expression of *Gsc2* at the age of E15.5. Our results also highlight the fact that Gsc2+ cells are not serotonergic, which was suggested earlier by Gottlieb *et al.* (1998). The co-expression of *Gsc2* and the GABAergic lineage could be replicated in different embryonic time points, including the adult brain, in order to establish *Gsc2* as not only caudal IPN marker but also as a GABAergic interneuron marker for that specific subtype of cells originating from the R1 area.

Co-expression of *Gsc2* with Other Transcription Factors

Results from the scRNAseq (Fig. 1C) predicted that *Gsc2* expressing cells would have a co-expression pattern at E13.5 with other genes encoding for transcription factors. One of these markers was *Sall3,* and cells expressing *Sall3* are located in the raphe area of the adult brain near-by the IPN (Morello *et al*., unpublished). Moreover, these Sall3+ cells seem to have their origin in the rV2 area in the hindbrain (Morello *et al*., unpublished). Our results show that *Gsc2* is not co-expressed with *Sall3* neither in the rV2 domain at E12.5 nor in the IPl at E15.5 in mice. This was seen in coronal sections in both time points used. For further investigation, expression of *Sall3* and *Gsc2* in cross-sections of

embryonic mice could have the potential to show this co-expression pattern in the rV2 domain. This further investigation would be relevant since our results showed a small population of Gsc2+ cells in the rV2 domain in cross-sections of R1 at E12.5 mice. Moreover, co-expression of *Gsc2* with other markers of the IPl or the IPc at different developmental time points (as shown by with *Otx2* by Ruiz-Reig *et al*., 2019) could reveal more about the nature of these Gsc2+ cells.

Notch Protein as a Determinant for GABAergic Cell Fate

Notch-Delta signalling is known to maintain the pool of neural progenitors during development (Chenn & McConnell, 1995), as well as control binary fate decision in the CNS in general (reviewed in Kageyama *et al*., 2008). In this study, our results suggest that Notch has a role in the binary fate decision, determining between GABAergic and glutamatergic fate during the development, similar to findings of Peng *et al.* (2007) in the ventral spinal cord. When cleavage of NICD by Psen1 was depleted, the expression of several GABAergic markers, *Gad1*, Gata3 and *Tal1*, was decreased in the rV2 domain at E12.5. The decrease of the *Tal1* expression was particularly evident in the mantle zone of the rV2 domain. Since the reduction of the expression signal was evident in both in situ hybridisation and immunohistochemistry, both mRNA production and protein production were affected by the inhibition of Notch signalling. Depletion of Notch did not, however, diminish the signal entirely, as can be seen in the general GABAergic marker *Gad1* and also in GABAergic marker Gata3. This would indicate that GABAergic fate selection during development is not entirely dependent on Notch signalling but has other factors in determining the neurotransmitter composition in the rV2 area of the developing mouse hindbrain. To see will the neurons take on glutamatergic fate due to depletion of Notch instead of GABA, is still uncertain. This question was tried to be answered in this study also, but the used glutamatergic markers did not function properly, as desired. Taken these results into consideration, Notch has a role in GABAergic fate selection of neurons during development in the rV2 domain of R1, but whether the depletion of Notch would increase the number of glutamatergic cells instead is still unknown.

Considerations

While these results were quite evident, some considerations need to be taken into account when deriving an interpretation of them. The in situ hybridisation and immunohistochemistry were

performed to multiple embryos to confirm the results, and the sections were chosen according to the anatomic guide provided by the Allen Developing Mouse Brain Atlas (2008). The protocols used for in situ hybridisation and immunohistochemistry were used and tested before by laboratory of Juha Partanen and proven to give accurate results. Laboratory equipment and reagents used were tested beforehand and showed to function correctly.

Our results showed the GABAergic nature of Gsc2+ cells and verified their developmental origin to the R1 region. To verify the GABAergic nature of Gsc2+ cells, future experiments with additional time points and orientation for different mice from different litters are needed. Moreover, in this study, a full-length copy of *Gsc2* gene was used to show the Gsc2+ cells, not the shorter version of the gene, which should be added to verification of the *Gsc2* expression. Additionally, our results from the E15.5 of co-expression with *Gsc2* and Gad67 did not show a strong co-expression pattern compared to the E12.5. In the future, results from the E15.5 should be replicated for verification of the GABAergic feature of Gsc2+ cells. The co-expression of *Gsc2* and *Sall3* was not shown in our in situ hybridisation results. The co-expression of these could be still possible. To prove the existence of Gsc2+/Sall3+ cells, experiments shown in this study should be replicated with different orientations and timepoints. Especially, experiments, including the timepoint E13.5, should be added since the scRNAseq results used were from that stage (Fig. 1). While it was shown earlier that *Gsc2* is related to REM-sleep by Funato *et al*. (2010), it is still unknown how exactly these particular cells contribute to that. The GABAergic nature shown in our results would indicate that these Gsc2+ cells are inhibiting some target in the CNS and contribute that way to the regulation of REM-sleep. Due to the location of Gsc2+ cells in the IPl, Gsc2+ cells might receive substance P afferent projections from the dorsal MHb and send GABAergic efferent projections to the pons area, and this way contribute to REM-sleep (hypothesis based on results from Haun *et al*., 1992; Funato *et al*., 2010; Quina *et al*., 2017). However, to see where and how the Gsc2+ cells are connected from the IPN to other areas in the brain, experiments related to projections of these specific neurons are needed to conduct.

As for the analysis of the neuronal phenotype after the inactivation of Notch signalling, our results showed the decrease of GABAergic marker expression in the rV2 domain of E12.5 mice when Notch signalling was depleted. These results align with results gained from the spinal cord by Peng *et al*. (2007) and hint a more expanded role of Notch signalling during development than shown before. Nevertheless, future experiments using different section orientation and additional markers,

glutamatergic markers and markers for Delta ligand expression, should be undertaken to fully understand the GABAergic and glutamatergic cell type diversification in the Psen1 mutant embryos. Also, additional embryonic stages can be included. Moreover, quantification of the reduction in GABAergic marker signal is needed to see the actual effect of this decrease in the neurons of rV2.

Conclusions

In this study, Notch signalling was shown to play a role in the development of GABAergic neurons in the rV2. These results could help to discover the mechanisms of determining the neurotransmitter identity in rV2 neurons. In addition to the study of specific neurotransmitter identity, a subpopulation of interneurons, validated in this study, was shown to originate from the R1 region and possess GABA as a neurotransmitter during development. These cells have a shown role in the regulation of REM-sleep (Funato *et al*., 2010), and the GABAergic nature revealed here could provide more information of the role of these cells in the caudal and lateral IPN networks related to REM-sleep. Furthermore, this study provides more evidence showing the developmental origin of subpopulations of interneurons. Hopefully, this study also fills some knowledge gaps related to the characterisation and mapping of the different cell populations within the nuclei in the brainstem.

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